Low Molecular Weight Protamine as Nontoxic Heparin/Low Molecular Weight Heparin Antidote (III): Preliminary In Vivo Evaluation of Efficacy and Toxicity Using a Canine Model

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ABSTRACT Heparin employed in cardiovascular surgeries often leads to a high incidence of bleeding complications. Protamine employed in heparin reversal, however, can cause severe adverse reactions. In an attempt to address this clinical problem, we developed low molecular weight protamine (LMWP) as a potentially effective and less toxic heparin antagonist. A homogeneous 1880-d peptide fragment, termed LMWP-TDSP5 and containing the amino acid sequence of VSRRRRRRGGRRRR, was derived directly from protamine by enzymatic digestion of protamine with thermolysin. *In vitro* studies demonstrated that TDSP5 was capable of neutralizing various anticoagulant functions of both heparin and commercial low molecular weight heparin preparations. In addition, TDSP5 exhibited significantly reduced crossreactivity toward mouse sera containing antiprotamine antibodies. TDSP5 showed a decrease in its potential in activating the complement system. All of these findings suggested the possibility of markedly reduced protamine toxicity for TDSP5.

In this article, we conducted preliminary in vivo studies to further demonstrate the feasibility and utility of using LMWP as a nontoxic clinical protamine substitute. Dogs were chosen as test animals because they were known to magnify the typical human response to protamine. By using a full spectra of biological and clinical assays for heparin, including the anti-IIa and anti-Xa chromogenic assays and the activated partial, thromboplastin time and TCT clotting assays, TDSP5 showed that it could completely neutralize all these different anticoagulant functions of heparin in dogs. Although administration of protamine in dogs produced a significant reduction in mean arterial blood pressure (-14.9 mm Hg) and elevation in pulmonary artery systolic pressure (+5.0 mm Hg), the use of TDSP5 in dogs did not elicit any statistically significant change in any of the variables measured. Furthermore, the use of LMWP also significantly reduced the protamine-induced transient thrombocytopenic and granulocytopenic responses. The white blood cell counts and platelet counts decreased to 82.1% and 60.0% of baseline, respectively, in dogs given intravenous protamine compared to 97.8% and 88.6% of baseline in dogs receiving TDSP5. These preliminary findings indicated that LMWP could potentially provide an effective and safe means to control both heparin- and protamine-induced complications.

Key Words: Heparin Neutralization, Protamine Toxicity, aPTT/TCT Heparin Clotting Assays, Anti-IIa/Anti-Xa Chromogenic Assays, Hemodynamic/Hematologic Responses

INTRODUCTION

Protamine is used routinely to reverse the anticoagulant action of heparin after cardiac or vascular surgeries. It is generally obtained from fish and consists of a group of heterogeneous peptides with an average molecular weight of 4500 d (1). Approximately 67% of the amino acid composition in protamine is arginine. The polycationic protamine combines with the polyanionic heparin through an electrostatic interaction, thereby neutralizing the anticoagulant functions of heparin.

The use of protamine in heparin reversal at times is associated with adverse reactions. The incidence of adverse reactions to protamine ranges from 0.06% to 10.7% and varies from urticaria to severe or fatal

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cardiac arrest (2-5). The mechanisms of these adverse hemodynamic and hematologic effects are manifold and include complement activation, thromboxane generation, histamine release, nitric oxide production, and antibody production (6). In general, undesirable anaphylactoid-type reactions, such as systemic hypotension, bradycardia, pulmonary artery hypertension, thrombocytopenia, and neutropenia, are produced by the nonimmunogenic pathway, where protamine crosslinks with heparin to form large heparin-protamine complexes (HPC) with network structures (7,8), and leads to subsequent activation of the classical pathway of the complement system (9). Several investigators have indicated that the larger the size of HPC, the more toxic are these crosslinked complexes (10-12). The activation products, anaphylatoxins (ie, C3a, C4a, and C5a), are some of the potential mediators involved in protamine-induced systemic hypotension and pulmonary hypertension. In addition, the crosslinking property of protamine has also been reported to contribute to its other toxicity, such as induction of platelet aggregation (13) and thrombin inhibition (14). On the other hand, anaphylactic-type reactions produced by the immunoglobulin-mediated pathway are primarily attributed to both the antigenicity (ie, the ability of a substance to be recognized by an antibody) and immunogenicity (the ability of a substance to induce antibody production) of protamine (2-5).

Our laboratory has pioneered an approach in developing low molecular weight protamine (LMWP) as a potential nontoxic heparin antidote. This chain-shortened LMWP is derived directly from native protamine to contain the arginine-rich, heparin-neutralizing domain in heparin, whereas the crosslinking and antigenic/immunogenic properties of protamine are reduced. Presented herein is a sequence of 3 publications describing our research on the development of this LMWP analogue. In 2 previous articles, 2 major LMWP preparations, termed TDSP4 and TDSP5, have been purified and characterized (15). In addition, their in vitro efficacy in neutralizing the anticoagulant functions of heparin and low molecular weight heparin (LMWH) and toxicity with regard to complement activation and crossreactivity toward mouse antiprotamine antibodies have been examined (16). In this third and last article of this series, preliminary in vivo studies of both the efficacy and toxicity of primarily the TDSP5 fragment using a canine model will be presented.

MATERIALS AND METHODS

Materials

Heparin sodium injection (1000 USP units/mL) was purchased from Elkins-Sinn, Inc (Cherry Hill, NJ). Protamine sulfate (10 mg/mL) was purchased from Eli Lilly (Indianapolis, IN). LMWP-TDSP5 fragment was prepared by digestion of native protamine with thermolysin and then purified by using a heparin column, according to the procedures described previously (15). Heparin, protamine, and LMWP solutions were prepared with 0.9% NaCl. All the materials used in this article and their resources were described in the previous articles in this series (15,16).

Assays

Details of the activated partial, thromboplastin time (aPTT), anti-IIa, and anti-Xa heparin assays were described in the previous publications (15,16). Measurements of the activated clotting time (ACT), thrombin clotting time (TCT), white blood cell (WBC) count, and platelet (PLT) count were conducted according to the procedures discussed in a previous publication (17). ACT measurements were carried out on whole blood specimens immediately after their withdrawal (2 mL) into celite-containing tubes, and were measured using a Hemochron 801 (International Technidyne, Edison, NJ) coagulation monitor. TCT measurements were performed on plasma samples using a fibrometer manufactured by BBL Microbiology Systems (Cockeysville, MD) and Fibrindex human thrombin reagent (Ortho Diagnostics, Raritan, NJ). WBC and PLT counts were determined using a manual hemocytometer and a dilutional red blood cell lysis method provided by the manufacturer (Unopette; Becton Dickinson Co, Rutherford, NJ).

In vivo Experiments

Fifteen healthy male random-source, mixed-breed dogs weighing 9 to 18 kg were used in this study. The animals were distributed into 3 groups (Groups #1, 2, 3). Group #1 consisted of 4 dogs used as controls; these dogs were given 2 mL of 0.9% NaCl after heparin administration. Group #2 consisted of 8

dogs to which protamine was administered after heparin was administered. To minimize the number of dogs involved in the in vivo experiments, the Group #2 animals included the 4 control dogs (after an appropriate washout period to allow for their recovery) plus an additional 4 new dogs. Group #3 consisted of 7 dogs for the testing of heparin reversal with LMWP (ie, TDSP5).

Animals were anesthetized with 30 mg/kg sodium pentobarbital, intubated, and maintained on isoflurane and oxygen during the study. Hydration was maintained with lactated Ringer's solution given intravenously as an initial 20 mL/kg bolus followed by continuous 10 mL/kg/h infusion for the remainder of the experiment. All animals were housed and cared for at the University of Michigan's Unit for Laboratory Animal Medicine under the direction of a veterinarian according to guidelines of the "Principles of Laboratory Animal Care" (National Society for Medical Research) and "Guide for the Care and the Use of Laboratory Animals" (NIH Publication No 86-23, revised 1985).

Each dog received 100 IU/kg intravenous porcine intestine heparin, followed 30 minutes later by 2 mL of 0.9% NaCl, 1 mg/kg of standard protamine, and 2.2 mg/kg of LMWP-TDSP5 for the Group #1, Group #2, and Group #3 dogs, respectively. (Note: The dose of heparin was adjusted for the weight of the dogs and is comparable to that used clinically in humans. The dose of protamine was based on the stoichiometric neutralization dose with 1 mg: 100 U (protamine:heparin) (18), whereas the dose of LMWP to achieve a complete neutralization of the anticoagulant effect of heparin was determined previously) (16). These compounds were administered intravenously into the femoral vein of the dogs over 10 seconds to maximize the hemodynamic responses. Hemodynamic parameters monitored included systemic mean arterial blood pressure (MAP) and pulmonary artery systolic pressure (PAS)-the two most indicative parameters in monitoring protamine-induced responses. The MAP was measured by means of a carotid artery catheter, whereas the PAS was monitored using an oximetric Swan-Ganz catheter (Abbott Laboratories,

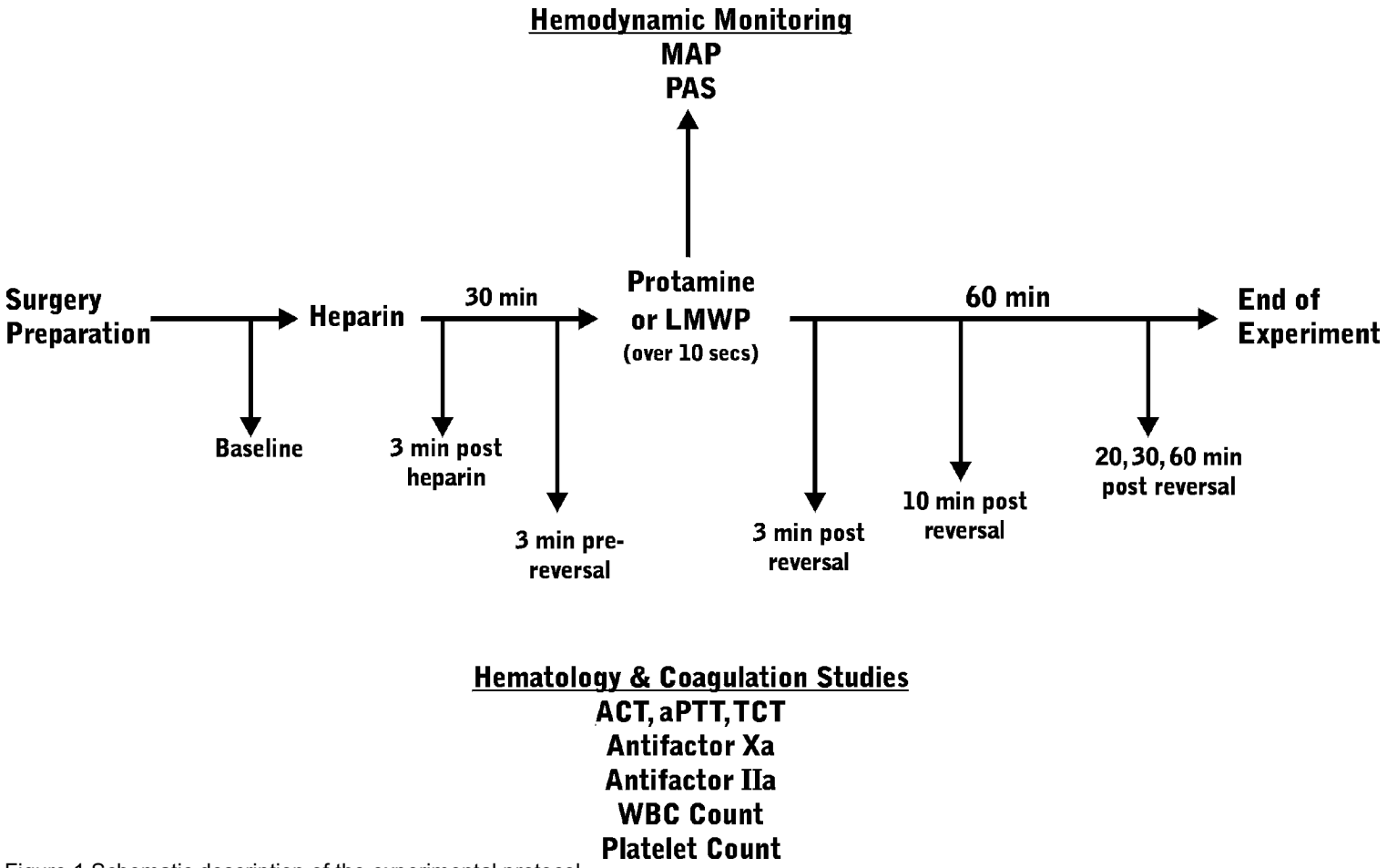

Figure 1 Schematic description of the experimental protocol.

North Chicago, IL) placed in the right femoral vein. All data were collected and analyzed by an online computer program (Workbench, Strawberry Tree, Inc, Sunnyvale, CA) that allowed continuous monitoring of these hemodynamic parameters. Measurements were recorded at baseline, 3 minutes after heparin administration, 3 minutes before heparin reversal, every second for 6 minutes after heparin reversal, and then at 10, 20, 30, and 60 minutes after heparin reversal.

Coagulation and hematologic studies were performed on venous blood withdrawn from the left femoral venous line. Samples were obtained at baseline, 3 minutes after heparin administration, 3 minutes before heparin reversal, and then at 3, 10, 20, 30, and 60 minutes after reversal. The coagulation studies included ACT, aPTT, TCT, anti-IIa activity, and anti-Xa activity. Hematologic parameters monitored included red blood cell, WBC, and PLT counts. To clarify the experimental procedures, a schematic description of the timeline of the experiments was presented in Figure 1.

Data were expressed as mean ± standard deviation. Comparisons of data between groups were made by use of 2-tailed Student *t* test where appropriate. The level of probability for statistical significance was established at $P \leq .05$.

RESULTS AND DISCUSSION

The dog was selected as the animal model for this in vivo feasibility study because dogs are known to magnify the typical human responses noted with protamine reversal of heparin (4). Conahan and coworkers reported that the cardiovascular effects of protamine appeared to be more benign in humans than in dogs (19). Our studies (17) and those of other investigators (11,12) showed that intravenous administration of protamine to dogs elicited significant hemodynamic responses. Therefore, the deliberate selection of the most sensitive animal model for this study was to magnify the detection of any possible adverse reactions associated with LMWP for heparin reversal. Accordingly, if LMWP does not yield any significant hemodynamic responses in dogs, it should be reasonable to assume that LMWP would not pose serious toxic effects in human beings.

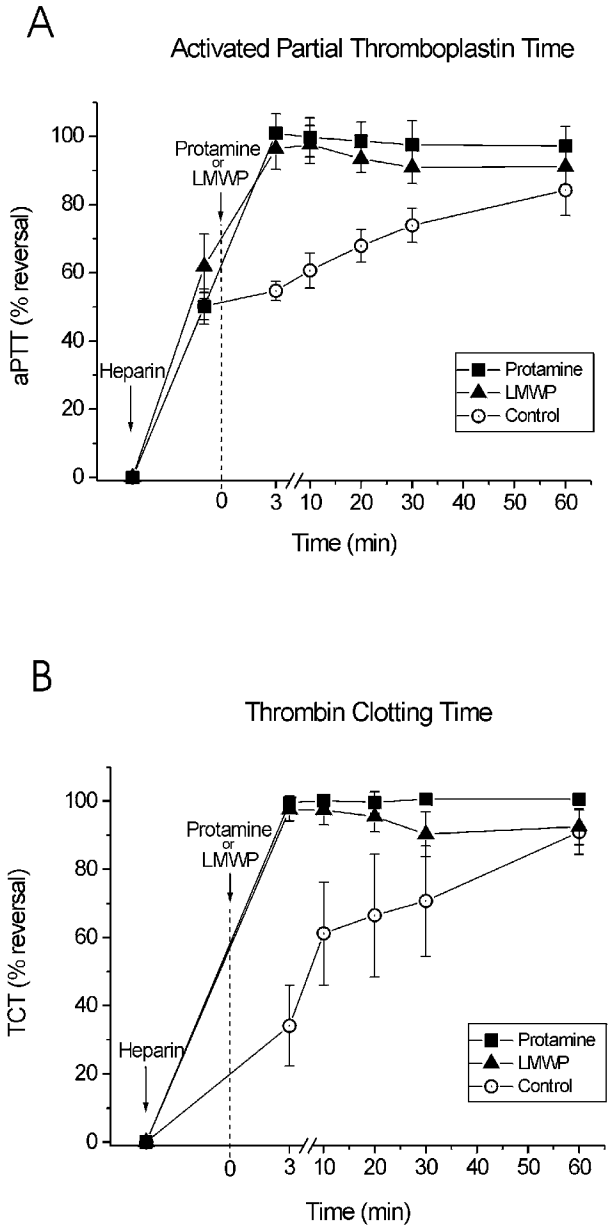

Figure 2 In vivo heparin neutralization as measured by the (A) aPTT and (B) TCT heparin-clotting assay. The data were normalized to 0% activity before heparin administration and 100% activity after heparin neutralization. The data were presented as the mean ± SD. For experimental details, please see the Materials and Methods section of this article.

Neutralization of heparin anticoagulant activities was measured by various biological and clinical assays including the ACT, aPTT, and TCT clotting assays, and the anti-IIa and anti-Xa chromogenic assays. As described previously, the aPTT assay measured heparin activity on a variety of coagulation factors in the intrinsic pathway of the coagulation cascade, whereas the TCT assay measures the thrombin activity more specifically. On the other hand, the

anti-IIa and anti-Xa chromogenic assays measured primarily the antithrombin III-mediated inhibition of thrombin (IIa) and coagulation factor Xa (15). Therefore, a combination of these assays would provide a full-spectra assessment of the effectiveness of the heparin-neutralizing ability by either protamine or LMWP. Figure 2 showed the plasma disappearance of heparin aPTT and TCT activities in the 3 animal groups under investigation. A complete neutralization of the aPTT activity (Figure 2A) by protamine (100.9 ± 5.7%) and by TDSP5 (96.3 ± 6.0%) was observed at the 3-minute mark after their administration. The difference between these 2 levels of neutralization was not statistically significant. Similarly, complete neutralization of the TCT activity (Figure 2B) was also observed by protamine (99.5 ± 1.9%) and TDSP5 (97.5 ± 3.4%) at the 3-minute mark. Alternatively, heparin neutralization monitored by the ACT method, which represented a general "whole blood" clotting assay similar in principle to that of the "plasma" aPTT assay, yielded similar results (data not shown) as seen in Figure 2.

Heparin neutralization measured by the anti-Xa and anti-IIa chromogenic assay was depicted in Figures 3A and 3B, respectively. The control group showed a similar heparin clearance rate relative to that observed by the aPTT assay (Figure 2). As seen, the anti-Xa and anti-IIa activities of heparin were completely neutralized by both protamine (97.5% ± 3.3% and 96.6% ± 6.8% reversal of anti-Xa and anti-IIa activity, respectively) and TDSP5 (96.5% ± 0.6% and 98.0% ± 2.6% reversal of anti-Xa and anti-IIa activity, respectively) at the 3-minute mark. None of the differences in heparin neutralization by protamine and TDSP5 were statistically significant. A combination of these results indicated that TDSP5 was fully capable of completely neutralizing the whole spectrum of heparin-induced anticoagulant functions.

It was not clear as to why heparin anticoagulant activities measured by both the clotting and chromogenic assays seen in Figures 2 and 3 resurged at a later stage of the experiments (eg, at the 30-minute mark in Figure 2B) after their reversal by LMWP but not with protamine. A number of investigators have observed the clinical phenomenon of so-called "heparin rebound" (20), wherein heparin activities recur in the circulation following their reversal with protamine. Several hypotheses have been proposed to explain this phenomenon. One mechanism suggests that a metabolic removal of protamine takes place, that somehow elicits the reappearance of heparin in the circulation (21), whereas another mechanism suggests that the loss of protamine excess leads to the instability of the heparin-protamine complexes with liberation of heparin (10). In either case, it makes examining the pharmacokinetic properties of the heparin-LMWP complexes necessary. Further studies in this direction are in progress in our laboratory.

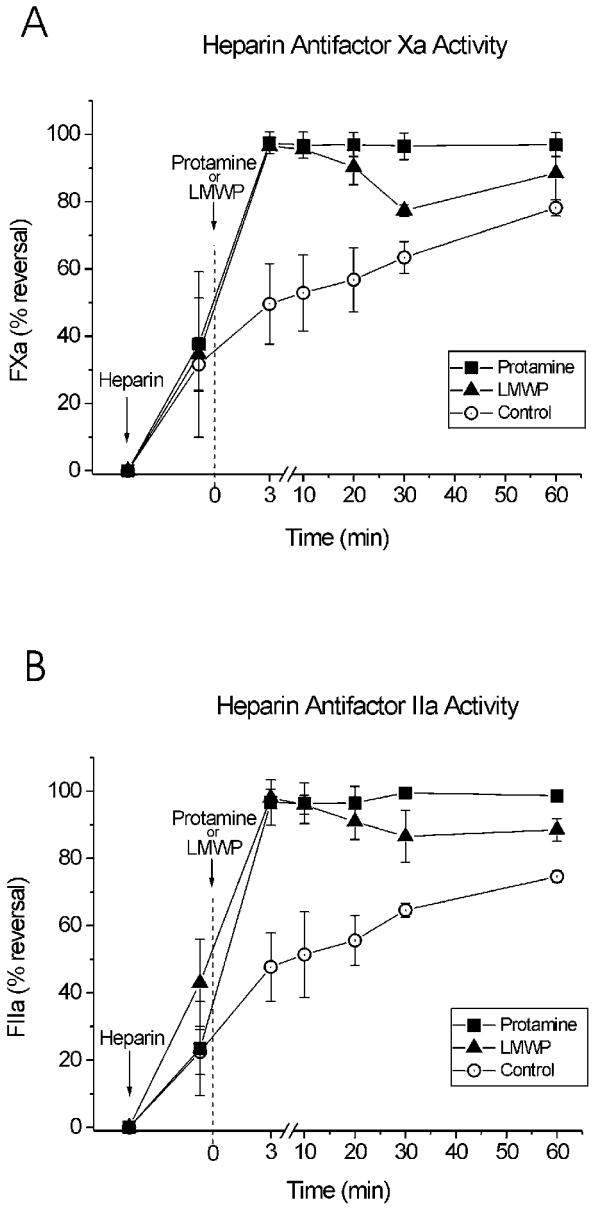

Figure 3 In vivo heparin neutralization as measured by the (A) anti-Xa and (B) anti-IIa heparin chromogenic assay. The data were normalized to 0% activity before heparin administration and 100% activity after heparin neutralization. The data were presented as the mean ± SD. For experimental details, please see the Materials and Methods section of this article. FIIa indicates activated clotting factor IIa (thrombin); FXa indicates activated clotting factor Xa.

The degree of adverse responses to protamine correlates with the rate of protamine administration, with the most rapid infusion inducing the most severe adverse effects (22). Therefore, a rapid bolus injection was used to elicit the characteristic protamine-induced responses. The MAP and the PAS pressure, which were among the most significant parameters in signaling protamine-induced adverse responses, were monitored to evaluate the hemodynamic toxic effects of both protamine and LMWP. Although the dog model has been used extensively for toxicity testing of pharmaceutical compounds, the extent and frequency of protamine-induced hemodynamic changes can be variable and may be associated with dosing and breed selection (10,11,23). In this preliminary study, 3 of 8 Group #2 dogs demonstrated typical and marked hemodynamic responses, whereas 1 of 8 exhibited an atypical but severe and dramatic drop of 77 mm Hg in MAP after receiving intravenous protamine. As shown in Figure 4, statistically significant changes ($P < .05$) in MAP were observed between the 3- to 4-minute marks (average maximum decrease of 14.9 mm Hg occurred at 3.5 minutes) (Figure 4A), as well as in PAS between the 4- to 5.5-minute marks (average maximum increase of 5 mm Hg occurred at 5 minutes) (Figure 4B) in the protamine group when compared to the control group. In contrast, the use of TDSP5 for heparin neutralization in the Group #3 dogs did not elicit any protamine-related hemodynamic responses. None of the MAP or PAS changes observed at any time interval was statistically significant when the data were compared to those at time zero or to the control animals (Group #1).

Another strong indicator for protamine-induced toxicity was its effect on blood cell counts. Numerous investigators noted that transient thrombocytopenia and granulocytopenia were manifested in animals and patients after protamine sulfate reversal of heparin activity (2-5). Although these phenomena could not be accounted for in terms of a single mechanism, their occurrence was largely attributed to the activation of the complement system (24,25). In addition, PLT aggregation could also be the result of crosslinking of adjacent PLTs by protamine (13) or be correlated to the development of hypotension by protamine reversal of heparin activity (24). Because the chain-shortened LMWP was deprived of the crosslinking ability and the capability of forming the large heparin-protamine complexes-a necessity to bind C1q and initiate complement activation (17,26)]-it was anticipated that using LMWP would alleviate protamine-associated thrombocytopenic and granulocytopenic responses.

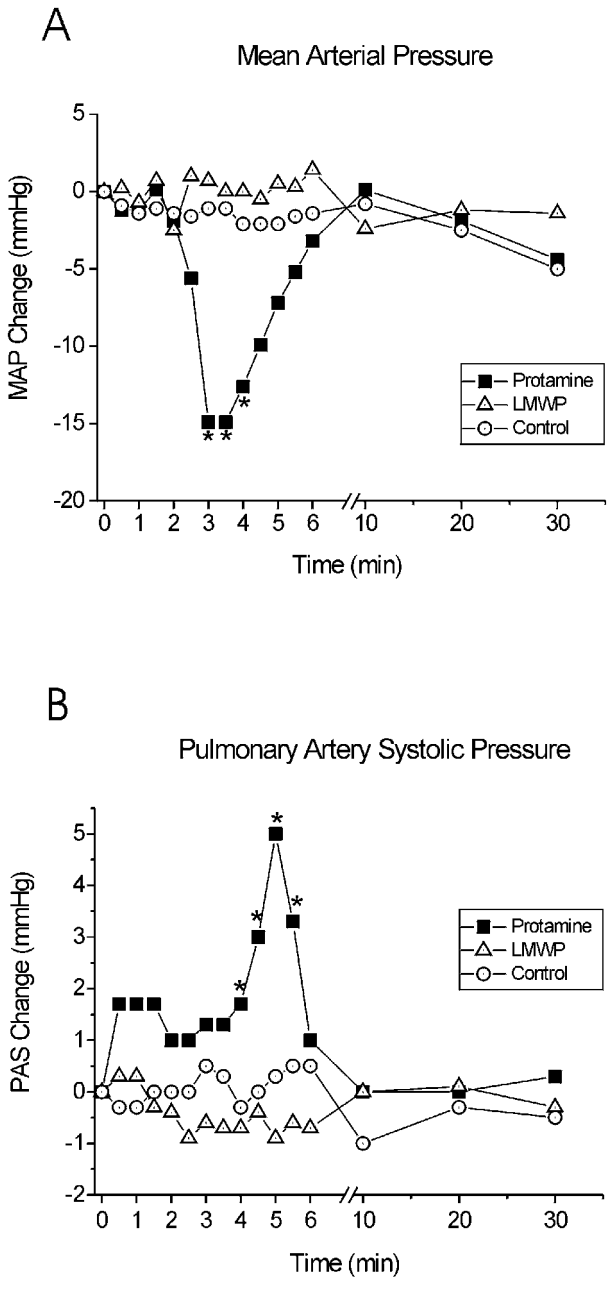

Figure 4 Hemodynamic changes in dogs. Hemodynamic parameters measured include (A) mean arterial pressure (MAP) and (B) pulmonary artery systolic pressure (PAS). Mean data were presented. *$P < .05$ from the control group. For experimental details, please see the Materials and Methods section of this article.

Table 1. Maximum Changes in White Blood Cell Counts and Platelet Counts in Dogs Following Heparin Reversal with Protamine and Low Molecular Weight Protamine (LMWP) (mean ± SD)

White Blood Cell (WBC) Counts		
	Before Treatment	**After Treatment**
Group #1 dogs (n = 4) (Control)	8,323 ± 896 (100%)	7,852 ± 1,384 (94.3%)
Group #2 dogs (n = 8) (With intravenous protamine)	8,494 ± 2,886 (100%)	6,970 ± 4,100 (82.1%)
Group #3 dogs (n = 7) (With intravenous LMWP)	6,993 ± 1,712 (100%)	6,836 ± 1,631 (97.8%)
Platelet (PLT) Counts		
	Before Treatment	**After Treatment**
Group #1 dogs (n = 4) (Control)	271,750 ± 74,625 (100%)	227,000 ± 39,640 (83.5%)
Group #2 dogs (n = 8) (With intravenous protamine)	253,000 ± 88,378 (100%)	151,778 ± 56,102 (60.0%)
Group #3 dogs (n = 7) (With intravenous LMWP)	280,429 ± 70,069 (100%)	248,429 ± 90,290 (88.6%)

Table 1 summarizes the maximum changes in WBC and PLT counts, which occurred predominantly at the 3-minute mark after heparin reversal with either protamine or LMWP. As expected, Group #2 animals receiving intravenous administration of protamine exhibited a significant and yet typical reduction of both WBC and PLT counts. A decline by 18% and 41% from the baseline values in the WBC and PLT counts, respectively, was observed in Group #2 protamine dogs. However, the WBC and PLT counts in Group #3 dogs at 3 minutes after reversal revealed that LMWP was no more toxic than normal saline. Compared to the baseline values, both groups of dogs (Group #1 control and Group #3 LMWP) exhibited a statistically insignificant reduction in WBC counts (6% and 2%, respectively) but a significant reduction in the PLT counts (16% and 11%, respectively). A common consequence of surgical procedures and blood loss is a decrease in PLT numbers and may account for the thrombocytopenic state. All the changes in WBC and PLT counts were transient; these cell counts returned to their baseline values before the conclusion of the experiments (ie, ~60 minutes). Compounding the results of Figure 4 and Table 1, it seems reasonable to suggest that TPSD5 significantly lacks both the hemodynamic and hematologic toxicity seen in protamine.

CONCLUSIONS

The objective of our investigation is to test the hypothesis that a chain-shortened peptide fragment derived from native protamine-LMWP-can retain the entire heparin-neutralizing function of protamine but with reduced toxic effects of protamine. In this last article of a 3-article series, we conducted preliminary in vivo studies using a canine model that was hypersensitive to signal protamine-induced responses to examine both the efficacy and toxicity of TDSP5. The results suggested that TDSP5 could completely neutralize the full spectrum of heparin anticoagulant functions in vivo. In addition, unlike protamine, using LMWP for heparin reversal did not elicit any protamine-related hemodynamic and hematologic responses. Based on these findings, it seems reasonable to suggest that LMWP could be an effective and less toxic heparin antidote. A precaution, however, should be made with regard to the validity of our assumption that LMWP would not induce any protamine-related response. That is, the canine model employed in the current study could only produce the type of protamine toxicity that was triggered by the nonimmunological pathway. Future studies will be conducted to demonstrate if LMWP could also exhibit reduced protamine toxicity mediated by the immunologic pathway by using animals that are presensitized with protamine to elicit the production of antiprotamine antibodies in such animals.

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